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PRINCIPAL INVESTIGATOR: Richard J. Santen, M.D.

CONTRACTING ORGANIZATION: University of Virginia
Charlottesville, Virginia 22904

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Introduction:

Deprivation of estrogen, called Endocrine Therapy (ET), is commonly used to treat women with estrogen receptor (ER) positive breast cancer. Resistance to ET occurs in many women after about 18 months treatment. Upregulation of growth factor pathways mediated by the 21 kDa Ras GTPase protein may contribute to resistance to ET. A novel Ras antagonist, farnesylthiosalicylate (FTS), causes Ras downregulation with concomitant abrogation of growth factor pathways. We tested the ability of FTS, which was complexed to a cyclodextrin moiety for solubility, to reduce the growth of ER positive breast cancer cells that were resistant to ET. FTS prevented growth of ER positive breast cancer cells by increasing apoptosis and reducing proliferation. Accompanying loss of cell growth was a significant reduction in the response to estrogen. The loss of estrogen response may have been due to an observed loss of ER protein in response FTS treatment. FTS might be causing reduced cell growth in part by increasing turnover of the ER in ER positive breast cancer cells. FTS was additive with Doxorubicin in vitro. We suggest that the FTS should enter preclinical trials against ER positive breast cancer.

Body of Report:

Methods

Reagents: FTS and cyclodextrin (CD) were generously donated by Thyreos Corporation, New Jersey. FTS-CD complex was prepared according to instructions from Thyreos; CD alone and PBS buffer controls were prepared exactly the same way. ICI 182,780 was generously donated by Astra-Zeneca, United Kingdom. E2 was obtained from Steraloids (Newport, Rhode Island). Cell Death Detection ELISA and Cell Proliferation ELISA were from Roche. Neutral Red was from Aldrich. IMEM was from Biosource and Fetal Bovine Serum (FBS) from Gibco. DextranT70 was from Pharmacia and Charcoal (NoritA) from Sigma.

Cell Culture: To remove E2 and related metabolites from serum, FBS was treated with Dextran and charcoal. Dextran Charcoal Coated stripped serum (DCC) was prepared. Inactivated FBS (500 ml) was added to 5 g of washed charcoal and 167 mg of DextranT70. The mixture was stirred at 4C overnight and the Dextran/charcoal pelleted out. This was repeated with fresh Dextran/charcoal two more times. In the final step, the FBS was spun twice at 33,000 rpm in an ultracentrifuge and then filtered through a 0.1 μ m filter. The resulting Dextran/charcoal coated stripped serum is referred to as DCC.

MCF-7 cells were cultured in IMEM, glutamine and 5% FBS, LTED cells were cultured in phenol red free IMEM, glutamine and 5% DCC. Cell number was assayed by a modified neutral red method. To assay E2 dependent growth, MCF-7 cells were seeded into 96 well plates; the next day the medium was changed to IMEM DCC and 5 days later E2 was added in fresh IMEM/DCC. Three days later fresh E2 in IMEM/DCC was

added and cell number measured 2 days later. To measure response of LTED cells to E2, LTED cells were seeded into 96 well plates; the next day, media was changed to IMEM with just glutamine added. Seven days later, E2 plus 10^{-9} M ICI182,780 in fresh IMEM was added; three days after that fresh E2 was added in IMEM/ICI and 2 days later cell number was measured. To assay the effects of FTS-CD on cell number, proliferation and apoptosis, cells were seeded into 96 well plates and the next day drug was added in fresh medium. Three days later, fresh drug in medium was added and cell number, apoptosis and proliferation measured 2 days later.

Cell Extract Preparation and Western Blotting: Cells were seeded at 2 million cells per 10 cm diameter dish. The next day CD or FTS-CD was added and three days later the cells were harvested into RIPA buffer. Cell extracts were normalized and loaded onto 10% SDS-PAGE gels, transferred to PVDF membrane and probed with the indicated antibodies. The antibodies used were: 62A3 reactive against residues around serine 118 of the ER from Cell Signaling, Ab17 against the N-terminus of the ER, Ab20 against whole ER (both from Neomarkers), phosphoRb against phosphorylated residues 807 and 811 of Rb from Cell Signaling, C-15 against the C-terminus of Rb from Santa Cruz and Actin antibody from Sigma.

Results: (Task I) Breast cancers that are susceptible to Endocrine Therapy are usually ER positive and wild type p53. MCF-7 cells are a wild type p53, ER positive, E2 dependent cell line and are a generally accepted model for early stage ER positive breast cancer that

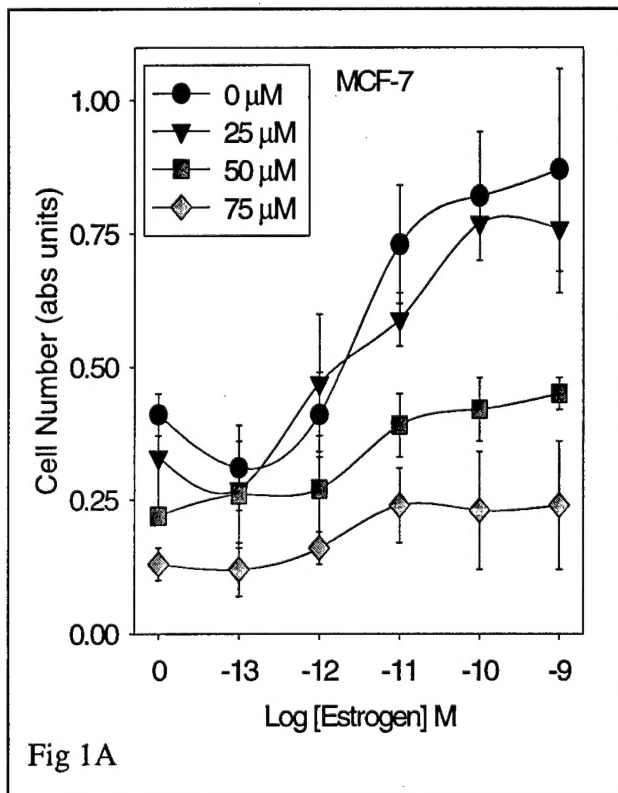


Figure 1: FTS suppresses E2 dependent breast cancer cell growth. MCF-7 (A) and LTED

is treated by Endocrine Therapy. LTED cells were generated from MCF-7 cells by depriving them of E2 over several months. LTED cells have many of the characteristics of breast cancers that regrow in the low E2 serum levels after Endocrine Therapy. Upregulation of growth factor and ER pathways occurs during regrowth after Endocrine Therapy. Therefore we tested whether a growth factor pathway inhibitor suppressed the growth of breast cancer cells in vitro. We assayed the effect of FTS, a Ras inhibitor, as the 21 kDa Ras protein is at the nexus of several important growth factor pathways, as well as being implicated in plasma membrane based ER signaling FTS suppressed the E2 dependent growth of MCF-7 cells (Figure 1A) with the greatest effects at 25 and 75 μ M.

(B) cells were grown under conditions where they demonstrate an E2 response as described in the methods. Increasing concentrations of FTS suppressed this E2 dependence. Representative of 2 experiments, mean and standard deviation of 4 samples are shown.

LTED cells display the characteristic maximal hypersensitive growth at two log lower E2 concentration than MCF-7 cells (Figure 1B). FTS started suppressing LTED growth at 25 μ M and there was a paradoxical slight stimulation of growth at 75 μ M FTS. There was little growth of either cell line in the absence of E2 under these conditions. These data support previous observations that E2 dependent ER signaling can pass through Ras *

We next compared free FTS to FTS complexed with CD. Complexing FTS with CD is required to solubilize the hydrophobic FTS molecule and would be essential for any possible future clinical use of FTS. FTS and FTS-CD were very similar in inhibiting cell growth of MCF-7 cells (Figure 2A). CD alone or Buffer vehicle had little effect on cell growth of MCF-7 and LTED cells (Figures 2B and 2C) under conditions where FTS-CD significantly abrogated cell growth. Even though LTED cells have higher MAPK activity than MCF-7 cells.

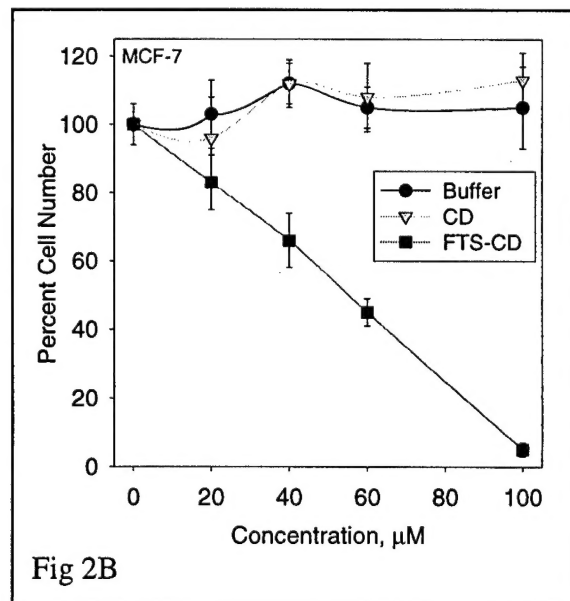
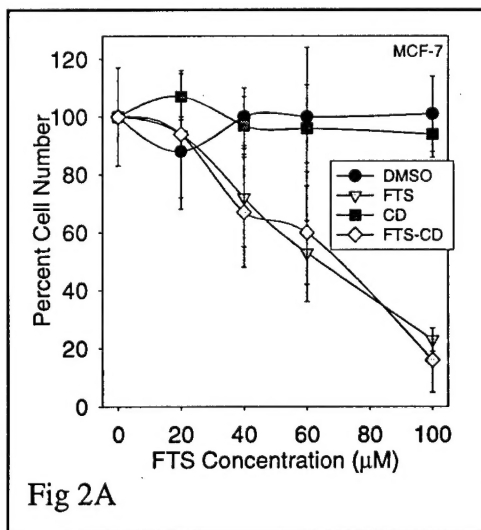
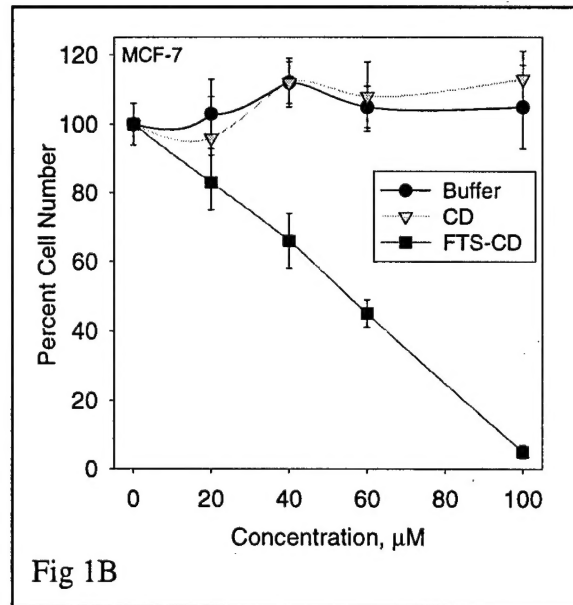


Figure 2: FTS and FTS-CD have similar growth inhibition profiles. **A.** MCF-7 cells were grown in the presence of FTS dissolved in DMSO, the equivalent volume of DMSO, FTS-CD dissolved in PBS or the equivalent amount of CD in PBS. Cells were treated for 5 days according to the methods. Representative of 2 experiments, mean and standard deviation of 4 samples are shown. **B.** and **C.** FTS-CD inhibits growth of both MCF-7 (**B**) and LTED (**C**) cells. Cells were

assayed according to the methods with either PBS, CD in PBS or FTS-CD in PBS added. Representative of 6 experiments, mean and standard deviation of 4 samples are shown.

Ras activity is required for cellular proliferation. Either underexpression or overexpression of Ras is capable of inducing an apoptotic response. Apoptosis was induced by between several hundred (not shown) to several thousand fold (Figures 3A and 3B) by 100 μ M FTS-CD, which is known to decrease the amount of cellular Ras. Proliferation was also reduced to very low amounts in both cell lines by FTS-CD, but not by CD or buffer alone (Figures 4A and 4B), affirming a reduction in an essential component of proliferative pathways.

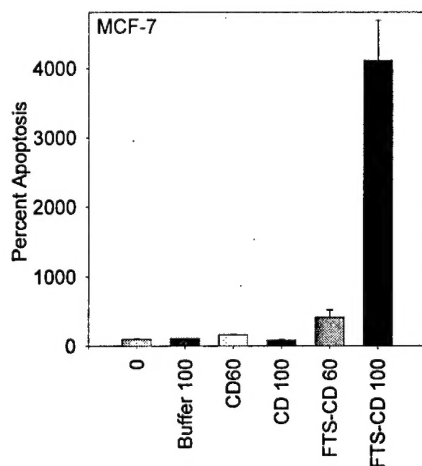
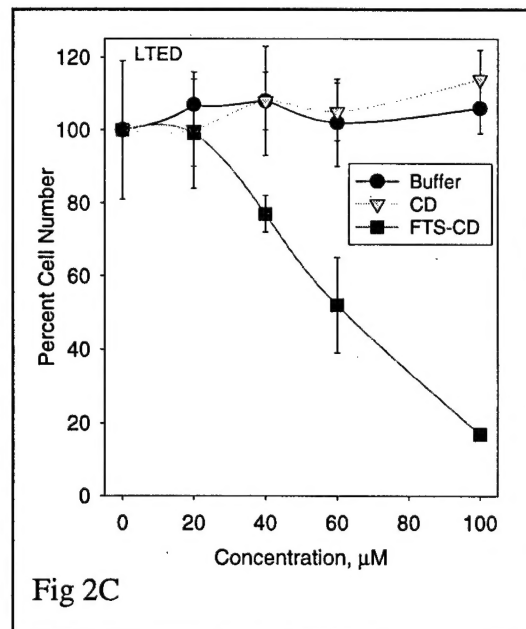


Fig 3A

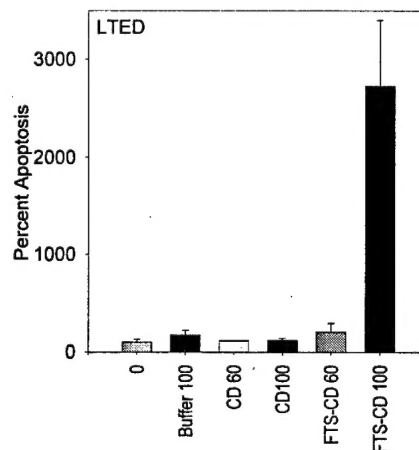


Fig 3B

Figure 3: FTS-CD increases apoptosis. MCF-7 (A) and LTED (B) cells were incubated with buffer (equivalent to 100 μ M FTS-CD), CD (equivalent to 60 or 100 μ M FTS-CD) or 60 or 100 μ M FTS-CD for five days as described in Methods. Apoptosis was measured by DNA nick site quantitative by ELISA. Representative of two experiments, mean and standard deviation of two samples are shown.

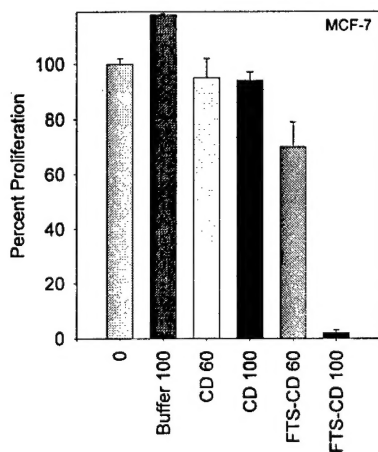


Fig 4A

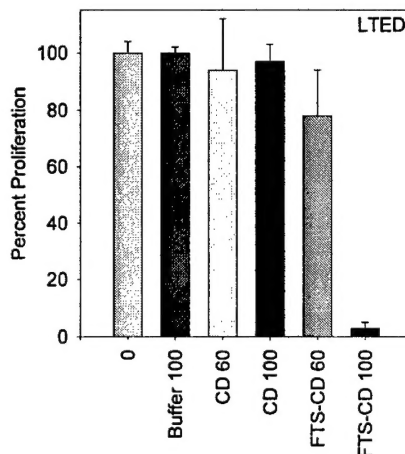


Fig 4B

Figure 4: FTS-CD reduces proliferation. MCF-7 (A) and LTED (B) cells were incubated with buffer, CD or FTS-CD for five days as described in Figure 3 legend. Proliferation was measured by BrDU incorporation. Representative of two experiments, mean and standard deviation of two samples are shown.

Downregulation of the ER is known to block E2 responsiveness, induce apoptosis and decrease proliferation of breast cancer cells. ER protein levels were downregulated after one to three days of FTS-CD treatment (Figure 5). This was confirmed with three different antibodies against different regions of the ER.

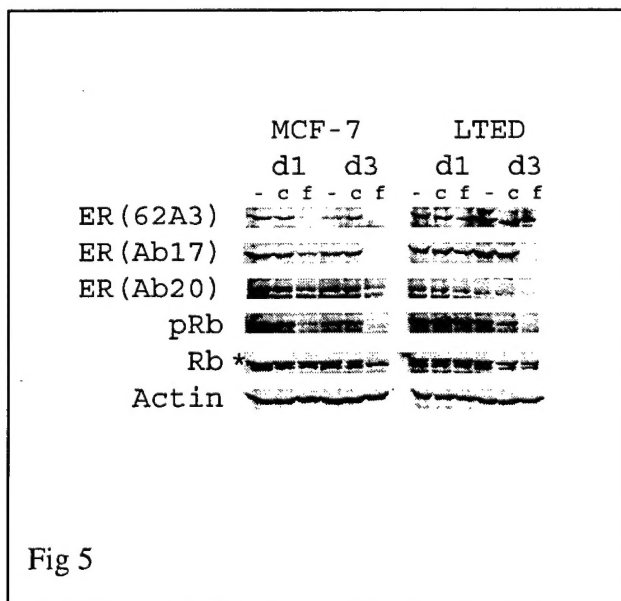


Fig 5

Figure 5: FTS-CD causes loss of the ER. MCF-7 (left six lanes) and LTED (right six lanes) cells were incubated with 100 μ M FTS-CD (f) or CD equivalent (c) or vehicle (-) for one (d1) or three (d3) days. The cells were harvested and proteins separated by SDS-PAGE, transferred to PVDF membrane and probed with indicated antibodies. Note that the Rb specific band is the upper of the two bands shown, asterixed. The lower Rb band appears to be non specific and does not align with the phosphoRb bands. Representative of two experiments.

Our data support a hypothesis that a major mechanism of FTS-CD on breast cancer cells is abrogation of E2 signaling, possibly by down-regulation of the ER protein. The tumor suppressor Rb is phosphorylated in cells

stimulated by E2. Phosphorylation of Rb allows activation of E2F1 and progression of the cell cycle. Rb phosphorylation was reduced by FTS-CD after three days (Figure 5). We also noted a decrease in total Rb in MCF-7 cells after 3 days of FTS-CD treatment but no decrease was observed in LTED cells.

The ability of FTS-CD to synergize with established anti-cancer drugs was tested. Notably Doxorubicin was additive with FTS-CD (Figure 6). Additivity occurred after 6 days growth at relatively low cell concentrations of 500 cells seeded per 96 plate well, but was not as marked at higher seeding densities of 750 and 1000 cells per well (not shown). We also tested additivity of FTS-CD with Tamoxifen, Paclitaxel and Cyclophosphamide prodrug but did not observe any reproducible additivity under the conditions used (not shown).

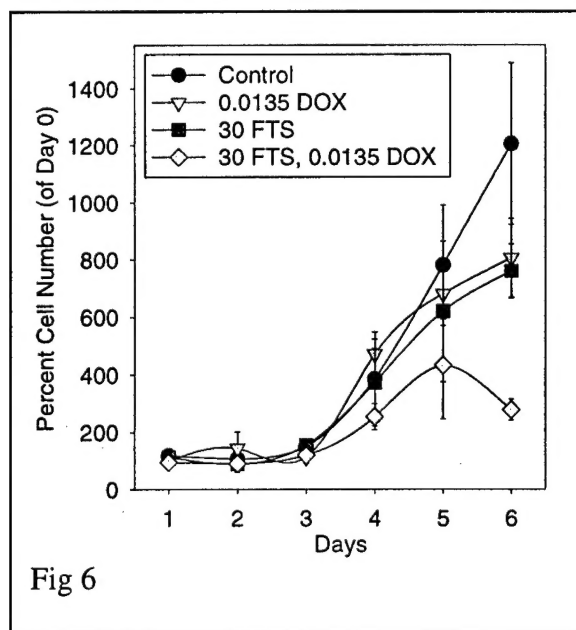


Figure 6: FTS-CD is additive with Doxorubicin. MCF-7 cells were seeded at 500 cells per well of a 96 well plate. The next day (day 0) cell number was measured as in methods and FTS-CD (30 mM) and/or Doxorubicin (0.0135 μ M) were added to the remaining wells as in the legend. Cell number was measured every day and fresh drug and media were added at Day 3. Shown are mean and standard deviation of four wells, expressed as a percentage of the number of cells measured at day 0. Representative of three experiments.

Key Research Accomplishments

The soluble FTS-CD complex clearly prevents growth of cellular models of E2 dependent (MCF-7) and E2 hypersensitive (LTED) breast cancers. The compound induces apoptosis up to several thousand fold and reduces proliferation to very low levels. The LTED cells have upregulated MAPK activity compared to parental MCF-7 cells addition of a specific MAPK inhibitor to LTED cells changes the E2 concentration that stimulates maximal cell growth. That is, they become less sensitive to E2 but growth is not completely suppressed. The FTS-CD suppresses growth but does not markedly shift E2 sensitivity. If the main effect of FTS-CD were solely mediated by MAPK, then the LTED cells with higher MAPK activity should have altered sensitivity to FTS-CD. The LTED and MCF-7 cells have the same sensitivity to FTS-CD so we conclude that a major cellular effect of FTS-CD is not through MAPK, but is on other pathways even though FTS-CD does change MAPK activity substantially in these cells. An exception to our

hypothesis is the apparent mild shift in hypersensitivity of LTED cells at 75 μ M FTS-CD. This observation implies that maximal inhibition of Ras activity in LTED cells, which have hyperactivated MAPK, might partially restore E2 sensitivity. Because we do not know why MAPK activity is upregulated in LTED cells, this anomolous observation is difficult to explain. However LTED cells do have more ER than parental MCF-7 cells, and it is possible that there is simply a different response because of this.

The observation that FTS-CD suppresses E2 dependent growth but does not shift hypersensitivity may be significant. Suppression of ER mediated growth suggested that ER signaling was being suppressed, rather than modified. Modification of ER responsiveness has been observed with the specific MAPK inhibitor, U0126, which changes the maximal E2 response of ER. Decreased levels of ER protein suggest the suppression of the E2 response might be though increased ER turnover. ER protein is normally degraded by proteasomal mechanisms and proteasomal inhibitors are very effective inhibitors of breast cancer cell growth in vitro (McPherson unpub.). The proteasomal pathway can be activated by oncogenic hyper activated Ras under certain conditions. Under other conditions, hyperactivated Ras can inhibit the proteasomal degradation of myc protein. Our observation that inhibition of Ras activates degradation pathways is consistent with a role for Ras as an inhibitor of proteasomal pathways in ER positive breast cancer cells. The alternative explanation for reduced ER levels on FTS-CD treatment is a reduction in transcription/translation of the ER. This hypothesis is also attractive because the ER has a half life of only a few hours. Our current data do not allow us to discriminate between these two possibilities.

Anthracyclines have a survival benefit of 3% over non-anthracycline chemotherapies of breast cancer at five years. FTS-CD is reproducibly additive with the anthracycline Doxorubicin in vitro. However this additivity was maximal at relatively lower cell densities. At lower cell densities there is less autocrine stimulation of Ras and MAPK, so pathway activity is lower than at higher cell densities. In cancer cells a relatively high density might occur as cells aggregate inappropriately. Whether this combination of drugs will be effective against breast cancer in vivo remains to be tested. FTS has not had significant toxicity in numerous preclinical trials. FTS is effective against breast cancer cells in vitro and is additive with an established anti-cancer drug. Therefore we suggest FTS-CD should undergo preclinical trials against breast cancer.

Reportable Outcomes: The data on in vitro inhibition of breast cancer cell growth is being prepared for submission for publication to the journal "Breast Cancer Research and Treatment".

Conclusions: These data provide compelling evidence that FTS causes a blockade of cell proliferation and an increase in apoptosis in breast cancer cells. These data show a high degree of promise that this agent will be effective in patients with breast cancer.

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